RESEARCH ARTICLE

Migrating cells mediate long-range WNT signaling

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ABSTRACT

In amniotes, it is widely accepted that WNTs secreted by the dorsal neural tube form a concentration gradient that regulates early somite patterning and myotome organization. Here we demonstrate in the chicken embryo that WNT protein is not secreted to act at a distance, but rather loaded onto migrating neural crest cells that deliver it to somites. Inhibiting neural crest migration or ablating their population has a profound impact on the WNT response in somites. Furthermore, we show that a central player in the efficient delivery of WNT to somites is the heparan sulfate proteoglycan GPC4, expressed by neural crest. Together, our data describe a novel mode of signaling whereby WNT proteins hitch a ride on migratory neural crest cells to pattern the somites at a distance from its source.

KEY WORDS: Somite, WNT, Dermomyotome, Chick embryo

INTRODUCTION

Somites are blocks of cells sequentially generated through the epithelialization of the segmental plate (Christ and Ordahl, 1995; Scaal and Christ, 2004). Shortly after their formation, somites differentiate along their dorsoventral axis into a ventral mesenchyme, the sclerotome and a dorsal epithelium termed the dermomyotome. The dermomyotome is then patterned along its mediolateral axis into a medial compartment termed the dorsomedial lip (DML), a central dermomyotome and a ventrolateral lip (VLL), each with distinct function and fate.

The DML is the most extensively studied dermomyotome subdomain. It plays a crucial role during the initial stage of muscle morphogenesis, during which the primitive muscle (the primary myotome) expands solely from the generation of muscle cells (myocytes) originating from the DML (Denetclaw et al., 2001; Gros et al., 2004; Kahane et al., 1998; Venters and Ordahl, 2002). This production of myocytes is dependent upon the transient activation of NOTCH signaling in selected epithelial progenitors present in this structure (Rios et al., 2011). Finally, the DML also plays a crucial role as a myotome-organizing center, as it expresses a secreted factor, WNT11, which acts as a directional cue for the elongation of early myocytes in the anteroposterior axis of the chick embryo. WNT11 mediates this effect through the evolutionarily conserved planar cell polarity (PCP) pathway (Gros et al., 2009).

The signals crucial for DML formation have been identified. Using WNT11 as a specific molecular marker of the DML, it was shown in the chick embryo that its expression is dependent upon WNT1 and/or WNT3a expressed by the dorsal neural tube, which trigger WNT11 expression through a WNT/β-catenin-dependent pathway (Gros et al., 2009; Marcelle et al., 1997). These results were confirmed and extended in mouse, where it was demonstrated that WNT1 and WNT3a exert their inducing activity on Wnt11 redundantly (Ikeya and Takada, 1998).

In chick and mouse embryos, the distance between the WNT1/3a source and the DML is considerable (~120 μm and ~90 μm, respectively; Fig. 1A-E,K-O). How WNT can act at such a distance from its source to pattern the medial somite compartment is unknown. A widely accepted view in the field is that WNTs form a concentration gradient from the dorsal neural tube that acts to pattern the somites at a distance (Capdevila et al., 1998; Fan et al., 1997; Marcelle et al., 1997; Munsterberg et al., 1995). However, the existence of this gradient has never been demonstrated experimentally. Importantly, WNT proteins are highly hydrophobic because of post-translational modifications by lipid adducts on highly conserved amino acid residues (Port and Basler, 2010; Takada et al., 2006; Willert et al., 2003) and as such are notoriously poorly mobile. These modifications attach WNTs to the lipid bilayers of membranes, restricting them to the secreting cells. In a number of cellular contexts, however, WNTs are known to act at a considerable distance from their source. This is accomplished through several molecular and cellular mechanisms. Heparan sulfate proteoglycans (HSPGs) of the glypican family, such as Dally and Dally-like protein (Dlp), allow the transfer of WNT from cell to cell, acting as a co-receptor for the ligand, thus allowing the formation of a gradient of WNT activity at a distance from the secreting cells (Yan and Lin, 2009). WNTs can also travel on membranous particles, called argosomes (Panáková et al., 2005), or within exosome-like vesicles (Korkut et al., 2009). Finally, although not formally demonstrated for WNTs, it is also possible that WNTs are transported at a distance similarly to Sonic hedgehog and the TGFβ family member Decapentaplegic, in which the receiving cells grow long cytoplasmic extensions that directly contact morphogen-secreting cells (Hsiung et al., 2005; Sanders et al., 2013).

Here we investigated the mode of signaling of WNTs from the dorsal neural tube to the DML, and uncovered a novel mechanism for long-range WNT signaling, whereby WNT expressed in the dorsal neural tube is loaded onto migrating cells (the neural crest cells) that physically transport the signal to the receiving cells of the DML. We also demonstrate the central role that the Dally-like molecule GPC4 plays in the transfer of the WNT signal from the presenting to the receiving cells.

RESULTS

WNT1 decorates the cell surface of migrating neural crest cells

Epithelial cells of the DML display long filopodia at their basal end that extend mediadorsally towards the dorsal neural tube (Rios et al., 2010, 2012). Although they may participate in the capture of signals from the environment, their length (~9-12 μm; our unpublished observations) is insufficient to reach the source of WNT in the roof plate. We therefore investigated alternative ways of WNT transfer from the dorsal neural tube to the DML.

The expression patterns of WNT1 and WNT3a have been extensively characterized during mouse and chicken embryonic
development (Hollyday et al., 1995; Marcelle et al., 1997; Parr et al., 1993). In mouse E9.5 embryos and in chick embryos at the equivalent
developmental stage (HH16), WNT1 and WNT3a mRNAs are specifically expressed in the roof plate of the neural tube. However, their transcripts are absent from migrating neural crest cells.

It is possible that, despite the lack of WNT transcript, WNT protein could be present on migrating neural crest cells. Indeed, at E9.5 in mouse and HH16 (E2.5) in chick embryos, WNT1 was detected, not only in the roof plate cells, but also at the cell membrane of early migrating neural crest cells, recognized by the expression of the neural crest-specific markers SOX10 in mouse and HNK1 in chick. WNT1 staining was also observed in the dorsal ectoderm, close to the roof plate (Fig. 1A-T). As neural crest cells migrate across the medial border of the dermomyotome and invade the sclerotome, WNT1 immunostaining decreases significantly. We attempted to confirm this finding for WNT3a; however, the specificity of the commercially available WNT3a antibodies was not sufficient to confidently assess its presence on migrating neural crest cells (data not shown).

**Neural crest migration is required for WNT11 expression and myotome organization**

This unexpected result prompted us to test whether neural crest cells play a role in the transport of WNT to somites. WNT11 mRNA is strongly expressed by all DML cells. Its expression is induced and maintained by WNT1/3a from the neural tube and it is therefore a very reliable readout of WNT1/3a activity in this structure (Marcelle et al., 1997). First, we ablated the neural crest cell population in half of the neural tube by electroporating the diphtheria toxin fragment A (DTA) under the control of a neural crest-specific promoter [U2-DTA; as previously demonstrated, this efficiently and specifically eliminates the neural crest population (Rios et al., 2011)]. To detect electroporated cells, we co-electroporated the DTA vector with a second vector containing EGFP under the control of a strong ubiquitous chick beta-actin promoter (GAGGS-GFP). On the electroporated side, the massive depletion of neural crest cells (as demonstrated by the loss of HNK1 staining) led to a significant decrease of WNT11 expression in the DML, compared with the control side (Fig. 2A-C; n=5/6). In parallel, a significant decrease of the WNT response was observed in the neighboring somite (supplementary material Fig. S1E-H) in comparison to a control (supplementary material Fig. S1A-D).

Second, we blocked the migration of neural crest cells using Dishevelled protein deleted of its DEP domain (DshΔDEP). The PCP pathway is required for the migration of neural crest cells in Xenopus (De Calisto et al., 2005) and DshΔDEP is a specific inhibitor of the PCP pathway (Rothbacher et al., 2000; Wallingford et al., 2000). We...
have previously shown that Dsh\(\Delta\)DEP expression in chick neural crest cells inhibits their migration without affecting their induction (Rios et al., 2011). Indeed, the electroporation of Dsh\(\Delta\)DEP-expressing vector into the dorsal neural tube, from which neural crest originates, led to a decrease in the number of migrating HNK1-expressing cells, and resulted in the strong reduction of \(WNT11\) expression in the DML of the electroporated side compared with the control side (Fig. 2H-J; \(n=4/4\)). Since the expression of \(WNT1\) and of \(WNT3a\) in the dorsal neural tube (which induce \(WNT11\) expression) are not affected by either the electroporation of U2-DTA or that of Dsh\(\Delta\)DEP in this structure (see figure 13 in the supplementary material of Rios et al., 2011) this demonstrates that neural crest migration is essential for the activation of \(WNT11\) in the DML.

A direct outcome of the decrease in \(WNT11\) expression in the DML should be the disorganization of the early myotome (Gros et al., 2009). Indeed, we observed that the ablation of the neural crest cell population (Fig. 2D-G) or the arrest of its migration (Fig. 2K-N) affected the orientated elongation of MyHC-expressing myocytes in the adjacent somites, compared with control somites (Fig. 2O-R).

Altogether, our data show that neural crest plays an essential role in the \(\beta\)-catenin-dependent WNT response in somites and the resulting expression of \(WNT11\), and that this has a direct consequence for the spatial organization of the early myotome.

**GPC4 in neural crest is necessary for WNT11 expression in the DML and for myotome organization**

Although neural crest cells are decorated by WNT at their cell surface, it is unclear how they deliver the ligand to the receiving cells of the somite. Frizzled (Fz) WNT receptors certainly play a role in this process: they are expressed by DML cells (Linker et al., 2005)
and functional testing suggests that FZ7 is the likely candidate for mediating WNT1 and WNT3a activities in this tissue (Gros et al., 2009). In addition, HSPG family members Dally and Dlp are known to bind WNT proteins and enhance the response of receiving cells to WNT (Yan and Lin, 2009). We investigated whether their vertebrate homologs are expressed at a time and place compatible with a role in transporting WNTs. Glypican 3 and glypican 5 precursors (GPC3 and GPC5) are the vertebrate homologs of Dally, whereas GPC1, GPC2, GPC4 [also known as knypek in zebrafish (Topczewski et al., 2001)] and GPC6 are the vertebrate homologs of Dlp. We analyzed the expression patterns of five out of the six chicken GPC genes (GPC2 was not tested) during embryonic development. Of these, only GPC4 displayed an expression pattern compatible with a role in neural crest in chick embryos. At stage HH16, its expression was restricted to the roof plate of the neural tube and to the early migrating neural crest cell population emanating from the neural tube. In addition, GPC4 was observed in the medial portion of the dermomyotome, including the DML (Fig. 3A-F).

We first examined whether GPC4 expressed by migrating neural crest cells plays a role on WNT11 expression in somites. To abrogate GPC4 function, we electroporated small interfering RNAs (siRNAs) directed against chick GPC4 into one half of the neural tube. This efficiently decreased the levels of endogenous GPC4 transcripts on the electroporated side of the roof plate and in the migrating neural crest cells (supplementary material Fig. S2A-C), whereas a control siRNA directed against the Luciferase gene had no effect on GPC4 expression (supplementary material Fig. S2D-E). The loss of GPC4 expression in migrating neural crest cells induced...
a significant decrease of WNT11 expression in the DML of somites on the electroporated side of the embryos (Fig. 3G-I, arrowheads) (n=6/8). We verified that this was not due to a decrease of WNT1 and WNT3a expression in the dorsal neural tube, to a decrease of WNT1 immunostaining in neural crest, or to their abnormal migration (supplementary material Fig. S2G-O). The electroporation of the control siRNA directed against Luciferase did not affect WNT11 expression (Fig. 3N-P, arrowheads; n=4/5).

The functional outcome of the downregulation of WNT11 after GPC4 knockdown should be a disorganization of the myotome. To verify this, we electroporated the GPC4 siRNA into the neural tube and examined the orientation of myocytes in adjacent somites. We observed a disorganization of the MyHC-expressing myocytes within the myotome in electroporated embryos (Fig. 3J-M), whereas control siRNAs had no effect on its organization (Fig. 3Q-T).

Altogether, these results show that GPC4 expressed by the neural crest plays a role in the signaling activity of WNT1 (and WNT3a) on DML cells, which results in WNT11 expression in this structure. In turn, this has a direct consequence for the orientation of myocytes in the myotome. Owing to the experimental protocol that we utilized (electroporation targets only a proportion of the neural crest and the siRNA technology leads to a decrease in expression and not to a complete knockout), we cannot determine whether GPC4 is necessary for, or merely enhances, WNT activity on the DML. In Drosophila, Dally and Dlp are not essential for wingless signal, but rather boost or sustain its activity (Franch-Marro et al., 2005), and it is therefore possible that, by analogy, GPC4 in the chick neural crest enhances WNT1/3a signaling on the DML.

**GPC4 is required in donor but not receiving cells for WNT11 activation**

Interestingly, GPC4 is also expressed in the DML. This observation gave us the opportunity to test whether GPC4 also plays a role in the receiving, responding cells of the DML. We electroporated the medial somites of E2.5 embryos with the GPC4 siRNA. As observed in the dorsal neural tube (see above), this led to a significant decrease in the level of GPC4 transcripts in the DML (Fig. 4G-I; n=5/5). However, this did not affect WNT11 mRNA expression in this structure (Fig. 4J-L; n=6/7), as compared with a Luciferase siRNA that did not alter GPC4 expression (Fig. 4A-C) nor WNT11 expression in the DML (Fig. 4D-F) (n=4/5). Altogether, this indicates that GPC4 does not play any significant role in the DML to regulate WNT11 expression. A similar function of Dlp restricted to the donor, and not in the receiving cells, has been described in the Drosophila embryo (Franch-Marro et al., 2005).

In this organism, it was suggested that Dally and Dlp have complementary, yet distinct, functions in Wingless signaling: Dally plays the role of co-receptor in the donor cell, whereas Dlp plays a role in transmitting the signal to the neighboring cell (Franch-Marro et al., 2005).

Although the experiments described above suggest a role for GPC4 in the transmission of WNTs, they did not address whether it has a role in cis. Previous studies have shown that WNT/β-catenin signaling plays an instructive role in the sensory neuronal fate of early migrating neural crest cells in mouse (Lee et al., 2004). To determine whether GPC4 may be implicated in WNT/β-catenin signaling in migrating neural crest as well, we electroporated a WNT reporter [TopFlash12X-d2GFP, which contains 12 TCF/LEF binding sites upstream of a destabilized EGFP (Rios et al., 2010)] in the dorsal neural tube and compared its activity after electroporation of the GPC4 siRNA or the control Luciferase siRNA. We observed a dramatic reduction in the number of GFP-expressing neural crest cells after electroporation of the GPC4 siRNA, indicating a decrease in canonical WNT signaling in the neural crest (Fig. 4P-R) as compared with controls (Fig. 4M-O). This indicates that, in addition to its role in the transmission of the WNT signal from the neural crest to the DML, GPC4 is also playing a role in mediating the β-catenin-dependent signaling of migrating neural crest. One day after co-electroporation with the WNT/β-catenin-dependent reporter (12XTopFlash2GFP) together with control Luciferase siRNA (M-O) or siRNA against GPC4 (P-R), embryos were stained for GFP to detect the activity of the reporter, and for RFP to detect electroporated cells. Scale bars: 50 μm.

**Fig. 4. GPC4 is required in donor but not receiving cells for WNT11 activation.** (A-L) siRNA against Luciferase (A-F) or chicken GPC4 (G-L) were electroporated into the DML of E2.5 chick embryos. One day later, embryos were sequentially processed by *in situ* hybridization for GPC4 (A-C,G-I) or WNT11 (D-F,J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of GPC4 (J-L) and by immunostaining for RFP (red) to detect electroporated cells.
activity of WNT1/3a in the neural crest cell population, presumably to regulate their neuronal fate.

**DISCUSSION**
Altogether, our results uncover a novel mechanism of long-range WNT signaling, whereby WNT ligands are loaded onto migrating neural crest cells that deliver their message at a distance. To improve the delivery process, neural crest cells express the Dally-like protein GPC4 that acts in trans to deliver the WNT ligand to the receiving cells in somites. This mode of WNT signaling is compatible with its known biochemical properties that impede long-distance signaling unless WNT is transported by ad hoc cellular or molecular mechanisms. There are higher levels of WNT1 protein on early emigrating neural crest cells, which decrease as they migrate away from the neural tube. This creates a gradient of WNT activity that, as in other signaling gradients, relies on the rate of synthesis, diffusion and degradation of the signal (Kicheva et al., 2007; Rogers and Schier, 2011). In *Drosophila*, the rate of degradation of WNT is driven by its endocytosis and targeting to lysosomes (Dubois et al., 2001) and this is the likely mechanism in the neural crest and DML. The rate of WNT diffusion from the neural tube to somites relies on the speed of migration of the neural crest cells away from the neural tube. This is a unique mode of dispersion since, in most systems studied, gradients of morphogens are established by the transfer of secreted signals from cell to cell within epithelia. Given the universality of cell migration and tissue movements during embryogenesis, it is possible that this mode of signal transduction represents a common mechanism for regulating differentiation or patterning programs within distant stem cell pools.

Our data reveal a novel role played by neural crest cells in the patterning of the dermomyotome and the direct impact that this has on the spatial organization of the myotome. For years, a widely accepted view was that neural crest comprises cells that only passively respond to signals from their environment. Recently, it was demonstrated that trunk neural crest cells play an active role in triggering myogenesis in selected epithelial progenitors in the DML and in maintaining the pool of PAX7-positive resident muscle progenitor cells during fetal life through expression of the ligands DELTA1 and neuregulin 1, respectively (Ho et al., 2011; Rios et al., 2012). Electroporations were performed by a first electroporation in the neural tube, followed 6 h later by electroporation of the DML of the somite. We constructed a TOPFLASH reporter (12TOPFLASH-ΔDep) in which 12 TCF/LEF binding sites (Rios et al., 2011). Double electroporations were performed by a first electroporation with pCAGGS-GFP to detect the electroporated cells as described (Rios et al., 2011). Together with earlier work showing that cephalic neural crest cells promote the formation of head muscles (Tzahor et al., 2003), this calls for a complete reassessment of the role of the neural crest in the patterning of their environment in general and of muscles in particular. From an evolutionary perspective, it is important to note that WNT reporters (this work) and NOTCH reporters (our unpublished observations) are also active in early migrating neural crest cells. Although their specific role in this cell population is unknown, this suggests that, during evolution, somites have ‘highjacked’ signals utilized for neural crest for their own purpose. Whether these signals are necessary for skeletal muscle differentiation and organization or are only utilized for the fine-tuning of these processes remains to be determined. It is however truly remarkable that, during evolution, two independent morphogenic movements in two distinct tissues have become so perfectly coordinated to generate such sophisticated signaling mechanisms.

**MATERIALS AND METHODS**

**Chicken electroporation**
Electroporations were performed as described (Rios et al., 2012). Plasmids were electroporated at 1 μg/μl final concentration in the electroporation mix.


